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Metacaspase gene function in the mushroom fungus *Schizophyllum commune*

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Metacaspase gene function in the mushroom fungus
Schizophyllum commune

By

Matthew P. Hanley

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Submitted in partial fulfillment
of the requirements for
Honors in the Department of Biological Sciences

UNION COLLEGE
June, 2011

Abstract

HANLEY, MATTHEW, P. Metacaspase function in the mushroom fungus *Schizophyllum commune*. Department of Biological Sciences, June 2011.

Abstract:

The overall goal of this project was to investigate the biological role of a putative metacaspase gene present in the mushroom fungus *Schizophyllum commune*. For this study, we have utilized a strain of *S. commune* that is unable to integrate DNA via the non-homologous end joining pathway. This forces transforming DNA to integrate homologously, as is required for the purposes of gene knockout. The gene *Scp1* encodes a likely member of the metacaspase protein family, which are suspected to have activity similar to caspases, the latter crucial to programmed cell death. A knockout construct containing a non-functional version of *Scp1* was previously generated in our laboratory. This DNA was then transformed into *Schizophyllum commune* in an attempt to knockout the native *Scp1* gene. At present a likely knockout (null) strain has been identified, and analysis by polymerase chain reaction has supported its status as a true knockout. A homozygous null mutant of *Scp1* will then be generated, and will be compared to a wild-type strain for any alterations in colony growth and/or mushroom development. The role(s) of other members of the metacaspase family will eventually be examined by a similar approach.

Acknowledgements

I would like to thank:

Dr. Stephen Horton, my thesis advisor, for his constant guidance and limitless patience.

Alyssa Goldberg and Nikhil Kothari, for their previous work and assistance.

Samantha Traver, my labmate, for her insight and company throughout the year.

My parents, Julie Hanley and Ed Charlebois, for their lifetime of support.

Dr. Lee L. Davenport, '37, for funding this project through the Davenport Undergraduate Research Fellowship.

The Internal Education Fund and the Department of Biological Sciences, for additional funding throughout the year.

Table of Contents

Abstract	ii
Acknowledgements	iii
Introduction	1
Methods and Materials	7
Strains and Media.....	7
Bioinformatics.....	8
Polymerase Chain Reaction.....	9
Gel Electrophoresis.....	9
DNA Concentration.....	9
Protoplast Generation.....	10
Transformation.....	13
DNA Isolation.....	14
Results and Discussion	15
Isolation of Ku70.....	15
The Ku70 Construct.....	17
The “Stuffer” Construct.....	17
The Knockout Construct.....	19
The Metacaspase-1 Knockout.....	21
Transformation into <i>S. commune</i>	22
Screening the Transformation Recipients.....	24
Negative Knockout Confirmation.....	25
Positive Knockout Confirmation.....	28

Generation of a Dikaryon.....	29
Works Cited	31

Introduction:

The cellular mechanism known as ~~apoptosis~~, or programmed cell death, has been the subject of vigorous investigation due to its importance in the development and homeostasis of multicellular organisms. Apoptosis is characterized by a range of morphological changes including cytoplasmic membrane blebbing, cytoplasm loss, chromatin condensation, and nuclear and genomic fragmentation (Kroemer *et al.*, 2000). This pathway can be initiated by a number of external and internal stimuli and is used by multicellular organisms to selectively remove damaged or unwanted cells (Kroemer *et al.*, 2000). In higher eukaryotes, this mechanism is initiated and regulated by a family of enzymatic proteins known as caspases, which function in a cascade to effect the morphological changes associated with apoptosis. Caspases are proteases which recognize and cleave adjacent cysteine and aspartate residues, a property from which their name is derived. Caspases involved in apoptosis fall into two categories: initiator caspases and executioner caspases. In humans, initiator caspases such as CASP9 and CASP10 cleave the pro-domains from inactive precursor caspases, causing a structural change resulting in their activation. Executioner caspases cleave various proteins within the target cell, thereby triggering programmed cell death (Cardone, 1998).

Though the mechanism of apoptosis has been most thoroughly characterized in complex eukaryotes it has been shown to occur in similar fashion in lower eukaryotic organisms (Lim *et al.*, 2007). In May 2000, Anthony G. Uren announced his discovery of a group of proteins present in plants, fungi, and protozoa that were structurally homologous to caspases; he named this family of proteins the metacaspases. (Uren *et al.*, 2000) Metacaspases are highly similar to caspases in portions of their genetic sequence

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and three-dimensional structure, as well as in the fact that they are synthesized as inactive proenzymes that must be cleaved in order to become activated. One key difference, however, is the active site; whereas caspases cleave adjacent cysteine-aspartic acid residues, metacaspases have been shown to cleave adjacent arginine-lysine residues (Uren *et al.*, 2000). Though the structure and function of caspases have been highly characterized in a number of organisms, the metacaspases have been studied with far less fervor. Though some initial research into the function of metacaspases has been conducted in single-celled yeasts, there has been essentially no work done to illuminate their role in multicellular fungi, which undergo a form of apoptosis. The metacaspases' strong homology with the apoptotic caspases, coupled with the fact that metacaspases are found in multicellular fungi in the absence of true caspases strongly suggests that metacaspases may be involved in directing multicellular fungal apoptosis. It is suspected that such a role would cause metacaspases to be involved with the elimination of decrepit or sterile cells, as well as certain aspects of the reproductive cycle, such as mushroom development.

A few functions for metacaspases have been identified in a few fungal species, some of which are reminiscent of functions carried out by caspases in more highly characterized species. Metacaspase knockout experiments conducted in the filamentous fungus *Podospora anserina* have produced overgrowth and extended life phenotypes, conditions which imply the loss of apoptosis, and are reminiscent of phenotypes seen in animal cancers, a disease which is highly correlated with the disruption of apoptosis (Hamann *et al.*, 2007). Similar overgrowth phenotypes were observed in metacaspase knockout strains of the yeast *Schizosaccharomyces pombe* (Lim *et al.*, 2007). A

knockout of the *YCP1* gene in the budding yeast *Saccharomyces cerevisiae*, which codes for metacaspase 1, produced a slow-growth phenotype which has been associated with cellular events characteristic of apoptosis, supporting theories that metacaspases serve a role analogous to caspases (Vachova and Palkova, 2007). A similar function was observed in the filamentous fungus *Aspergillus fumigatus* (Richie *et al.*, 2006). Of particular interest is a study conducted by Hoeberichts and Woltering which demonstrated that metacaspases are upregulated in *Arabidopsis thaliana* cells during infection by the fungal pathogen *Botrytis cinerea*. Furthermore, they demonstrated that this upregulation of metacaspase expression was correlated with the initiation of the apoptotic pathway in *Arabidopsis* (Hoeberichts and Woltering, 2002). Many of the metacaspase functions implied by the results of these studies suggest that metacaspases may be involved with apoptosis, supporting the hypothesis that they may serve a similar function in *Schizophyllum commune*.

This study specifically seeks to investigate the function of metacaspases in the mushroom fungus *Schizophyllum commune* for a number of reasons, including several characteristics which make it a good model organism and the potential for industrially relevant discoveries. *Schizophyllum commune* serves as a good model organism because it has a short life cycle of only two weeks, it is amenable to manipulation through traditional molecular biology techniques, and there are few ethical issues surrounding the sacrificing of a fungus for the sake of scientific discovery (Palmer and Horton, 2006). Furthermore, *S. commune* is similar to agriculturally useful mushroom fungi, such as *Agaricus bisporus* and pharmaceutically useful species such as *Cordyceps sinensis*, which may produce compounds with antitumor properties. (Bok *et al.*, 1999) Since

apoptosis is such an anciently conserved pathway, discoveries made in *Schizophyllum commune* may be relevant to mushroom development in these related commercially useful species. Contributing to its usefulness as a model organism, the H4-8 strain of *Schizophyllum commune* has had its genome successfully sequenced by the Joint Genome Institute, allowing for extensive use of bioinformatics analysis in the planning and design phases of the experiment (Ohm *et al.*, 2010).

In order to be able to recognize phenotypic changes which may arise as the result of a metacaspase knockout, it is important to understand the typical life cycle of our model, *Schizophyllum commune*. At any given time *S. commune* exists in one of two stages: a haploid phase during which the organism exists as a monokaryon, or a diploidic phase in which two monokaryons come together to form a dikaryon. When in the monokaryon form, the mating type of the cell is controlled by two genetic loci, the first of which has about 300 alleles and the second of which has about 90; this extensive variation allows for nearly 28,000 different mating types (Kothe, 2010). When two monokaryons of different mating type come together they fuse their cell walls, forming pores through which can pass the nucleus of either cell. The two nuclei co-localize and initiate a process known as conjugate nuclear division by which both nuclei are replicated within one cell. Following certain environmental triggers such as changes in light, moisture or temperature mushroom development may be triggered and a process known as karyogamy may be initiated. Karyogamy refers to the fusion of two nuclei in one cell and the subsequent division of this cell into monokaryotic spore cells. (Horton and Palmer, 2006)

When conducting knockout experiments it would be useful to have access to what is known as a “knockout-ready” strain. Such a strain would have been modified in such a way that it facilitates genetic knockouts, this can be done by modifying the DNA repair mechanisms of a particular strain. Such a strain of *Schizopyllum commune* was generated by a Dutch research group (de Jong *et al.*, 2009) who directed the knocking out of the DNA-repair gene *ku80*. This gene codes for the repair protein Ku80 which functions in a heterodimer with the protein Ku70 to direct the repair mechanism known as Non-homologous End Joining (NHEJ), or Ectopic End Joining (Li, 2011). Non-homologous End Joining is the mechanism by which double-stranded breaks in the genome can be repaired without the need for a homologous template. NHEJ occurs in the overwhelming majority of integration events and causes the target DNA fragment to be integrated into the genome randomly. NHEJ is the counterpart pathway to the mechanism known as Homologous End Joining, a much less common mechanism which requires the existence of a homologous template in the genome. Through this pathway, target DNA fragments are integrated in a “like-seeks-like” manner due to the homologous template requirement. Through this mechanism, a functional exogenous gene fragment aligns with its damaged equivalent in the genome and replaces it by means of a double crossover event. (Gu *et al.*, 1997)

The *Ku80* knockout ready strain, named $\Delta Ku80$, makes use of these counterpart pathways to facilitate further knockout experiments. By knocking out *ku80*, the *Ku80/Ku70* heterodimer has been rendered non-functional, preventing this strain from undergoing Non-homologous End Joining. This modification reduces the total number of DNA integration events, but forces all events that do occur to take place by the

Homologous End Joining pathway. This characteristic makes it particularly useful for gene knockout experiments because a non-functional experimental version of a target gene can be transformed into cells of this strain and in some instances replace the functional endogenous copy, resulting in a knockout.

This study seeks to make use of this *ΔKu80* strain to knockout the Metacaspase 1 gene, (*Scp1*), in *Schizophyllum commune*, with the goal of determining its biological function. My working hypothesis is that a loss of Metacaspase 1 will have effects on mushroom development, possibly manifested as a slow-growth phenotype or as the lack of mushroom development altogether. The *ΔKu80* strain will be transformed with a non-functional modified version of *Scp1*, transformants will be screened by PCR, and any knockouts will be manipulated through traditional genetics to generate a double-knockout dikaryon individual which will be observed throughout its life cycle.

Materials and Methods:

Strains and Media

The following strains were used in the generation and analysis of the *Scp1*-knockout strain. *S. commune* strain H4-8 is a homokaryotic, non-fruiting strain which has had its genome sequenced. Strain T-26 is highly similar, though it has not had its genome sequenced. The T-26 strain is Tryptophan-negative; it lacks the gene (*Trp1*; Munoz-Rivas *et al.*, 1986) that conveys the ability to produce the essential amino acid Tryptophan (Trp), rendering them unable to grow unless placed on a medium which contains Tryptophan. This deficiency was used as a selectable marker; all transformation recipients were initially Trp-negative, but the transformation construct contained a copy of the *Trp1* gene, ensuring that only those individuals that took up a copy of the clone would be able to proliferate. The $\Delta Ku80$ strain was provided by de Jong *et al.*; it has been modified in such a way that it has had its *Ku80* gene knocked out, rendering it unable to integrate DNA via the non-homologous end joining pathway (de Jong, 2010). A Trp- derivative of this strain was used in the generation of the *Scp1* knockout.

Organism	Strain Designation	Characteristics
<i>S. commune</i>	H4-8	Homokaryon, Trp+, Genetically Unmodified
<i>S. commune</i>	T-26	Homokaryon, Trp-, Genetically Unmodified
<i>S. commune</i>	$\Delta Ku80$	Homokaryon, Trp+, Deficient in <i>ku80</i>
<i>S. commune</i>	Sko2-14	Homokaryon, Trp-, Genetically Unmodified

Table 1. Strains and growth conditions of fungi used in this study.

Bioinformatics

Experimental design was carried out *in silico* using a number of freeware programs available through the internet. Previously, the genomic location of the Metacaspase-1 gene was determined using the Joint Genome Institute's open genome browser to access the sequence of the *Schizophyllum commune* genome. (<http://genome.jgi-psf.org/Schco1/Schco1.home.html>) This genome browser was used to extract the sequence of the Metacaspase-1 gene, which was used in subsequent planning phases, such as PCR primer design and restriction endonuclease digest. The freeware program Primer3, provided by the University of Massachusetts (<http://biotools.umassmed.edu/cgi-bin/primer3plus/primer3plus.cgi>) was used to design primer pairs for use in Polymerase Chain Reaction. The primers used in the generation of the knockout and subsequent screening steps are listed below.

Name	Direction	Sequence (5'-3')	Length (bp)	Purpose
SCPKO_U1	Forward	GTATGAGAAGGGCCGTACACTC	22	Negative SCP1 knockout confirmation
SCPKO_D2	Reverse	GCTACACAAGAGGGTGTCAGTG	22	Negative SCP1 knockout confirmation
SCPKO_U3	Forward	GTAGCTTTGGTTGTGTAGGATGC	23	Negative SCP1 knockout confirmation
SCPKO_D4	Reverse	GACAGCCAGAACCAGATACTCAC	23	Negative SCP1 knockout confirmation
SCPKO_U5	Forward	GAGGGTATAAACTTGACATGCTGAC	25	Negative SCP1 knockout confirmation
SCPKO_D6	Reverse	GTTGAGTAGATGTACGGAAGGTCTG	25	Negative SCP1 knockout confirmation
SCPKO_U7	Forward	CTAGCCTTATCACTTGATTGGACAT	25	Positive SCP1 knockout confirmation
SCPKO_D8	Reverse	TACATCTGACAGACAATCCGTTTTA	25	Positive SCP1 knockout confirmation
SCPKO_U9	Forward	GTATGGAATGCGAAGCTAGTGTATT	25	Positive SCP1 knockout confirmation
SCPKO_D10	Reverse	CTATGTATCTCTCTATGAGCACCA	25	Positive SCP1 knockout confirmation
SCPKO_U11	Forward	GTATGAGAAGGGCCGTACACTC	22	Positive SCP1 knockout confirmation
SCPKO_D12	Reverse	GCGTCCAGGTACAATGAGTACA	22	Positive SCP1 knockout confirmation
KU70_U1	Forward	ATCTTCGTCTTGATTCTGCCTTC	22	Creation of a Ku70 Knockout Construct
KU70_D2	Reverse	GATATCATCTTCTCCCCATGA	22	Creation of a Ku70 Knockout Construct
KU70_U3	Forward	GCAAATGATCTTCGTCTTGATTTC	24	Creation of a Ku70 Knockout Construct
KU70_D4	Reverse	CAGTTTAGGTCGTTTAGGCATTG	23	Creation of a Ku70 Knockout Construct
KU70_U5	Forward	CGTCATAATGGTCACGCTTTT	21	Creation of a Ku70 Knockout Construct
KU70_D6	Reverse	GAGCCCAGATATCATCTTCTCC	21	Creation of a Ku70 Knockout Construct
DS70_U1	Forward	TCCAGTACCTGCTGGAGAACAAGGTGAGTAAATG	36	Creation of a Ku70 Knockout Construct
DS70_D2	Reverse	GCTACTGGCCACTGAACAGAGAGAGATCATCCTG	36	Creation of a Ku70 Knockout Construct
DS70_U3	Forward	TCGAGTACCTGCAAAATGGTAGATGGGGCTAAGAAT	36	Creation of a Ku70 Knockout Construct
DS70_D4	Reverse	CTACTGTGGCAATTCTAACGGACTGCTTTTCAACT	36	Creation of a Ku70 Knockout Construct

Table 2. Oligonucleotide Primer pairs used in confirmation of the knockout.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was used to amplify relevant DNA fragments at several stages of the study. PCR protocols varied by experiment, but all protocols were initiated with a hot start. In all cases, the primers described above we used at a concentration of 10 μ M. PCR was used primarily as a screening technique; transformants had their genomes screened for the presence or absence of the artificial knockout construct.

Gel Electrophoresis

The products of both PCR experiments and restriction digests were analyzed using gel electrophoresis. Agarose concentration varied slightly according to the sizes of the DNA fragments being analyzed, but all experiments used gels between 0.7% and 1.2% agarose. All gels were run with a kb ladder molecular weight marker (Promega) against which experimentally derived bands were compared. The bands produced by the kb ladder are, from top to bottom: 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb, .75kb, .5kb, .25kb.

DNA Concentration

Concentration measurements were made primarily with the use of the Nanodrop machine (Thermo Scientific). Nanodrop measurements were always made using 1 μ l of sample. In some instances, concentration measurements were estimated by comparing band intensity to a benchmark gel with bands corresponding to known concentrations.

Protoplast Generation

In order to transform the knockout construct into individuals of *Schizophyllum commune*, fungal protoplasts first had to be created. Protoplasts were made essentially by the protocol of (Horton and Raper, 1991). Protoplasts are fungal cells which have had their cell wall removed, and it is in this form that cells must be in order to be amenable to transformation. Colonies of the T26 strain of *S. commune* were grown on CYM+Trp plates until colonies were about 1.5 inches in diameter. Colonies selected to be converted into protoplasts then had half of their mass excised and placed into a sterile grinding cup. To this cup was added 100 ml of sterile liquid CYM+Trp media, this mixture was then ground at top speed for about 60 seconds and then poured into a sterile 500 ml flask. This flask was incubated at 30°C overnight on a rotary shaker operating at 200 rpm. The following day, after at least 12 hours had passed, the mixture was placed back into a sterile grinding cup and subjected to a 30-second grind at maximum speed. Cultures were then poured back into a sterile 500 ml flask and allowed to recover overnight at 37 °C. After at least 12 hours, the cultures were examined, those that were observed to have a cloudy liquid layer on their surface were determined to be contaminated and were discarded. The remaining cultures were transferred to 50ml conical tubes for subsequent steps. The cultures had their supernatants pipetted off and discarded, while 50 ml dH₂O were added to the remaining cells and mixed gently. The tubes were then centrifuged at 2000 rpm for 5 minutes. Following centrifugation, the supernatant was pipetted off and discarded, while 50 ml of 0.5 M MgSO₄ + 10 mM MOPS (3-(N-morpholino)propanesulfonic acid) was added to the remaining cells; this mixture was allowed to sit on the benchtop for 5 minutes. Following this, the mixture was again spun

in the centrifuge at 2000 rpm for 5 minutes. The supernatant was then pipetted off and discarded, while 50 ml 1 M MgSO_4 + 10mM MOPS was added, again this mixture was allowed to sit on the benchtop for 5 minutes. Following this 5-minute waiting period, the tube was again spun in the centrifuge at 2000 rpm for 5 minutes.

During this final 5-minute spin, the enzyme mixture used to degrade the fungal cell walls was prepared. 0.25 g of Driselase and 0.4 g B-D-Glucanase were dissolved in 10 mL of 0.5 M MgSO_4 + 10 mM HOPS. This mixture was filter sterilized and set aside until the final centrifugation was complete. Following the completion of the final centrifugation, the supernatant of the sample was pipetted off down to the 12 ml mark and discarded. To the remaining cell culture was added 3 ml of the enzyme mixture. This mixture was then gently poured into a sterile petri dish, ensuring that the bottom of the plate was evenly covered. These plates were then incubated at room temperature on a rotary shaker operating at less than 100 rpm for 6 hours. After the requisite 6 hours, the mixtures were observed macroscopically, they were observed to be brown, grainy and less viscous than they were before the 6 hours, indicating the degradation of the cell walls. The mixtures were examined microscopically to confirm the release of protoplasts, which appear as small spherical cells. The solution from each plate was then pipetted up and down several times to ensure even mixing, then transferred to a sterile 50 ml conical tube. The plate, and remaining cell material, was washed with 1.5 ml of 1M MgSO_4 + 10 mM MOPS, this wash was then added to the previously mentioned 50 ml conical tube, bringing the volume to about 15 ml. 11.25 ml dH_2O was then added to the tube and mixed gently, this mixture was then allowed to sit for 10 minutes. After this 10 minute incubation, the mixture was spun in a centrifuge at 1370 rpm for 5 minutes,

causing the cell wall debris to form a pellet. After centrifugation, all but 10 mls of the supernatant were pipetted off and transferred to a new sterile 50 ml conical tube and set aside. The pellet was then resuspended in the remaining 10 ml of supernatant and respun at 1370 rpm for 5 minutes. Following this spin, the new supernatant was pipetted off and added to the supernatant set aside in the previous step. This total volume of supernatant was then divided into 2 50 ml conical tubes. To each of these tubes was added 1M Sorbitol + 10 mM MOPS bringing the volume up to the 50 ml mark, this combination was then mixed gently and incubated for 10 minutes at room temperature. After the 10 minutes, the solution was centrifuged at 1790 rpm for 10 minutes, after which all but about a milliliter of the supernatant was removed and discarded. The left over pellet was resuspended in the remaining liquid and with a sterile transfer pipet all tubes containing the same strain were consolidated into a single 15 ml conical tube. The tubes were topped off with 1M Sorbitol + 10mM MOPS, bringing the volume to 15 ml. A hemocytometer was used to calculate the concentration of protoplasts, the number of cells within the 4x4 grid was counted, then multiplied by 25 and 10^4 and the volume of solution in the tube (15 ml), giving the total number of protoplasts isolated. This mixture was then centrifuged a final time at 1760 rpm for 10 minutes, after which as much of the supernatant was removed as possible without disturbing the pellet. The protoplast pellet was resuspended at a concentration of 1.3×10^8 per ml. To this protoplast solution was added 5 μ l 1M CaCl_2 / 100 μ l, and the resulting mixture was refrigerated overnight at 4°C.

Transformation of Protoplasts

This transformation procedure was dependent upon Polyethylene Glycol (PEG). The 50% PEG solution contains 50 g of PEG and 1 ml of 1M MOPS dissolved in dH₂O such that the final volume is brought to 100 ml. The Regeneration medium contains 20.5 g sucrose dissolved in 100 ml of liquid CYM. The following solutions were prepared in a sterile microfuge tube and allowed to chill on ice for 10 minutes: 54 µl test DNA, 3 µl sterile 1M CaCl₂, and 0.8 µl beta-mercaptoethanol, to which was added TE bringing the final volume to 60 µl. After this mixture had chilled, 100 µl of the protoplast solution were added, and the combination was mixed gently with a pipet tip. This solution was incubated on ice for 30 minutes. Following the incubation, 160 µl of the 50% PEG solution was added slowly down the inside of the tube, this mixture was incubated on ice for 10 minutes. The mixture was then pipetted gently up and down to mix the protoplasts, and then again incubated on ice for 10 minutes. Following this incubation, the entire mixture was added to 5 ml of liquid regeneration medium in a sterile plate. These plates were then incubated overnight at 30 °C. Following overnight incubation, 1 ml of the protoplast mixture was added to 4 ml of liquid CYM+Trp low melting point agarose, which was mixed evenly and poured into a petri plate. The top agarose was allowed to solidify, then the plates were inverted and incubated at 30 °C for 2 -4 days, allowing the individual transformants to grow into colonies.

DNA Isolation

Due to the potentially large number of transformation recipients that might be needed to be screened, traditional DNA isolation techniques were foregone in favor of a “Microwave Prep” technique. 2mm plugs were cut out of the fungal colonies to be screened, these were then placed into microcentrifuge tubes. These tubes were placed in a microwave beside a beaker containing 500 ml of water. The tubes were then microwaved at 100% power for 8 minutes with one 30 second intermission during which time the water in the beaker was replaced. The beaker with 500 ml of water was placed in the microwave to absorb any excess radiation and prevent damage to the microwave; the water was replaced with cooler water when it began to boil to prevent spillover inside the microwave. Following the 8 minutes of microwaving, 90 μ l of TE buffer were added to each tube, the tubes were vortexed to mix well and then spun down in a microcentrifuge. The supernatant was removed into new tubes that were stored in the refrigerator at 4 °C.

Results and Discussion:

Isolation of the *Ku70* Gene

Identification of the *Ku70* gene in *Schizophyllum commune* was accomplished using the JGI genome browser; the sequence of the *Ku70* gene had previously been identified, so a search for the keyword “*Ku70*” was sufficient to return the location of the gene. *Ku70* was found to be located on scaffold 9, spanning nucleotides 56974-59859. The sequence for the coding region of *Ku70* and 2 kb flanking DNA was extracted from the browser and used as the template for subsequent manipulations. The *Ku70* gene is comprised of 15 exons and 14 introns; the DNA sequence that was extracted from the genome was approximately 7 kb, including the 2kb flanking regions on either end of the coding region.



Figure 1. Joint Genome Institute genome browser showing the *Ku70* coding region in *Schizophyllum commune*. (http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=Schco1&position=scaffold_9:56974-59859)

The sequence extracted from the JGI database was then used to generate primer pairs for the purpose of isolating and amplifying *Ku70*. The sequence was inserted into

the program Primer3, which was used to generate three primer pairs specific to *Ku70*. These primer pairs were designed to amplify approximately 6 kb, corresponding to the *Ku70* coding region and about 1.5 kb flanking DNA on either side. Several optimization experiments were run to determine the most effective PCR reaction kit, annealing temperature, and runtime protocol. Ultimately, it was determined that the Qiagen HiFidelity Hotstar PCR reaction kit was the most effective for the amplification of *Ku70*. This kit makes use of the traditional Taq-polymerase enzyme that is commonly used in PCR, but it is supported by the addition of the Qiagen “Hotstar” enzyme, a specially isolated enzyme which has exonuclease activity in addition to its polymerase activity. This added function gives the enzyme proofreading ability and promotes high-fidelity transcripts in long DNA fragments; additionally, the exonuclease activity is alleged to prevent mispriming. With the inclusion of this Hotstar enzyme, it was determined that primer pair Ku70_U1/Ku70_D2 produced the most consistently distinct bands, indicating the consistent isolation and amplification of the *Ku70* gene. This primer pair was observed to work with equal accuracy at temperatures ranging from 48°C to 54°C. Results of the optimization PCR can be seen in Figure 2, below.

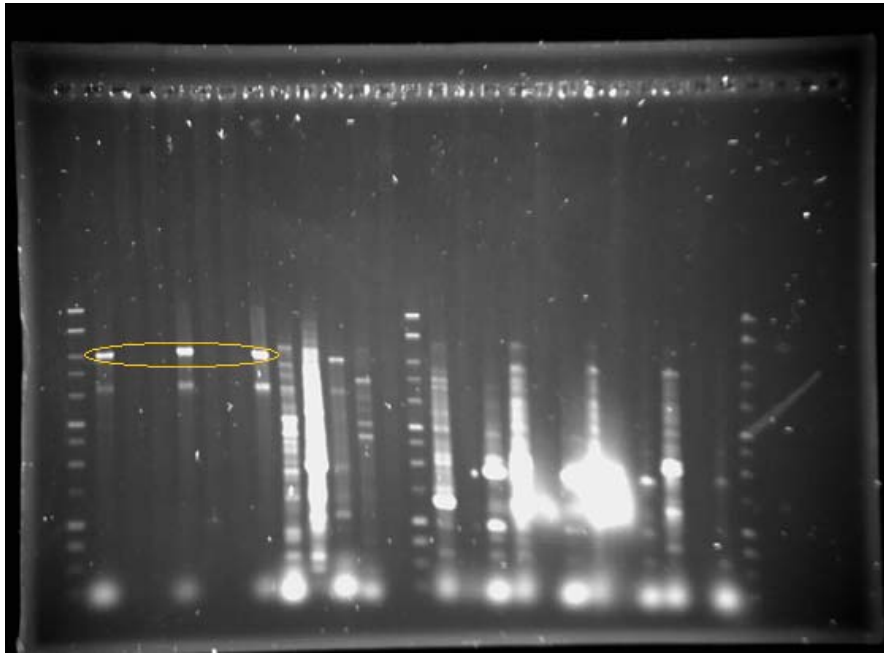


Figure 2. Indicated bands represent successful isolation and amplification of Ku70 from *Schizophyllum commune* genomic DNA. Bands correspond to the primer pair Ku70_U1/Ku70_D2 at 48, 50 and 52 °C from left to right.

The KU70 Construct

Once the *Ku70* gene had been successfully identified and isolated, it was used in the construction of a *Ku70* ligation vector. This vector was designed to be used in a later experiment where it would mixed with a vector containing a piece of “stuffer” DNA. Through restriction digest, these two vectors would be cut with the same restriction enzymes, generating homologous sticky ends, which would then, in a few instances, recombine to form a *Ku70* knockout construct. This vector, named pNAK1, was created in the Horton lab by a previous research student (Kothari, 2010). The vector is about 9.5 kb and contains a number of restriction enzyme sites which were used in the creation of the knockout construct. The vector is pictured below.

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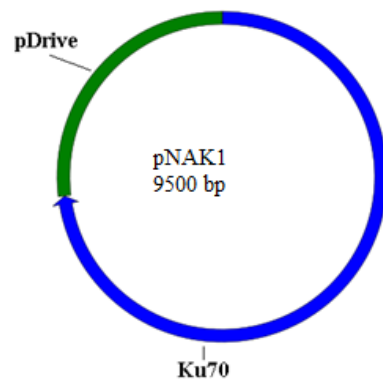


Figure 3. The cloning vector pNAK1, which is comprised of the pDrive vector into which has been inserted the *Ku70* gene. It should be noted that in addition to the *Ku70* gene being inserted into the vector, a small region of the pDrive vector (about 100bp) has been removed and discarded.

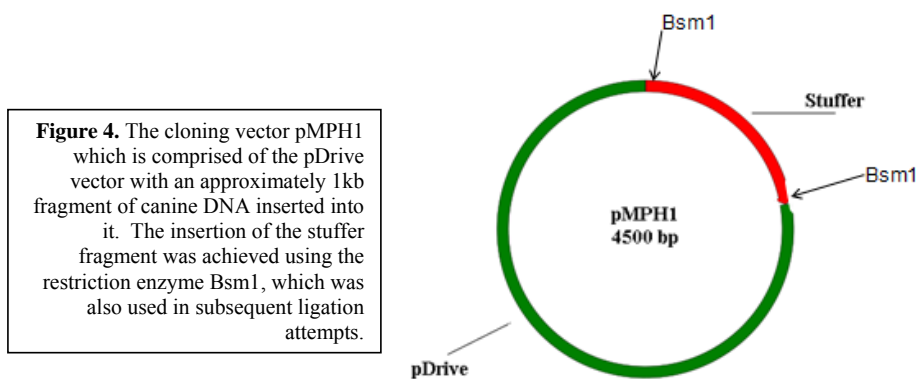
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The “Stuffer” Construct

In order to create a non-functional version of the *Ku70* gene to be used in later knockout experiments, it is necessary to disrupt the coding region of the gene. This is best done by removing a portion of the coding region and replacing it with a segment of non-coding, foreign DNA. In this experiment, a segment of the *Ku70* gene (within pNAK1) was removed and replaced with a segment of the canine *IGF-1r* gene, which codes for the Insulin-like Growth Factor receptor. This particular gene was chosen as a source of stuffer DNA because it had 0% homology with the *S. commune* genome, indicating that it would have no function in a *S. commune* cell if it were successfully introduced to the genome, as it would be in the case of a knockout. Primers were designed to specifically isolate and amplify an approximately 1000 bp region of this *IGF-1r* gene for use in the creation of a knockout construct. After a series of optimization experiments it was determined that the primer pairs DS_U13/DS_D14 and DS_U15/DS_D16 were able to isolate the stuffer fragment most successfully. Amplification was carried out with both of these primer pairs at 55°C using canine genomic DNA previously purified in the Horton lab.

The isolated stuffer fragment was then placed into a cloning vector of its own in anticipation of the assembly of the knockout construct. Again, the pDrive cloning vector and the dog stuffer fragment were digested with the restriction enzyme Bsm1 and placed into a 4°C water bath with a ligation buffer solution overnight to allow for recombination. Gel electrophoresis confirmed the creation of this stuffer-containing vector, which was named pMPH1.



The Knockout Construct

In order to make progress towards a *Ku70* knockout organism it was necessary to create a “knockout construct”. A knockout construct is a vector which contains the target gene, in this case *Ku70*, which has been disrupted by the stuffer fragment. This construct was attempted to be created by means of restriction endonuclease digestion and subsequent ligation. It has been previously mentioned that in the construction of the stuffer vector the stuffer fragment was digested with the restriction enzyme Bsm1. Since the stuffer fragment can be any section of DNA that does not have a function in the ultimate knockout organism, there was a certain degree of freedom in selecting the fragment to use. This fragment was specifically chosen because it could be easily

inserted into the coding region of *Ku70* by means of restriction enzyme digestion because *Ku70* also has two BsmI recognition sites within its coding region, about 1000 bp apart. In this way, pNAK1 could be digested with BsmI, removing a 1kb segment of DNA and leaving homologous ends for the stuffer fragment to ligate to. This is the approach used in the creation of the knockout construct; both pNAK1 and pMPH1 were digested with BsmI, causing the stuffer fragment in pMPH1 to be cut out of the pDrive vector and causing the previously 1kb segment of *Ku70* to be cut out of pNAK1. The solutions containing these fragments were then mixed and incubated at 4°C overnight with a ligation mixture with the goal of promoting recombination in a few instances such that the knockout construct would be created. The anticipated knockout construct vector, named pMPH2, is pictured below.

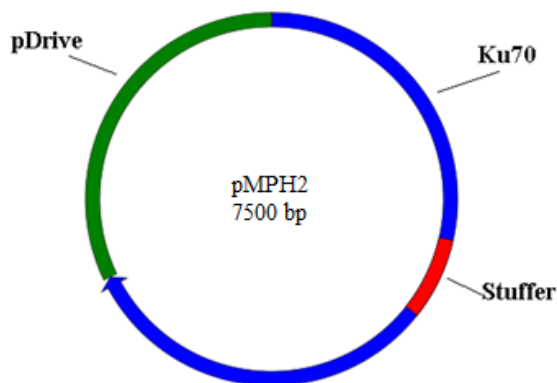


Figure 5. The hypothetical knockout construct, pMPH2. The insertion of the canine stuffer into the coding region of *Ku70*, along with the simultaneous removal of a segment of the coding region would render *Ku70* non-functional *in vivo*. Such a construct would be ready for transformation into suitable recipient strains of *S. commune*.

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Unfortunately, all attempts at creating this knockout construct were unsuccessful. Analysis with gel electrophoresis showed that all of the component DNA pieces were being correctly excised by restriction digest, but in no instances would the fragments recombine into the above conformation. Around this time, the Dutch research group led

by Jan de Jong published a paper revealing their successful creation of a *Ku80*-knockout strain of *Schizophyllum commune* using similar methods (de Jong *et al.*, 2010). As previously stated, *Ku70* and *Ku80* work together as a heterodimer and the loss of either protein results in the loss of the Non-homologous end joining pathway. The researchers responsible for this knockout strain were kind enough to supply our lab with a sample from which we could develop our own stock cultures; at this time it was decided that attempts at the construction of a *Ku70*-knockout organism should be abandoned and the established knockout strain should be used in alternate knockout experiments.

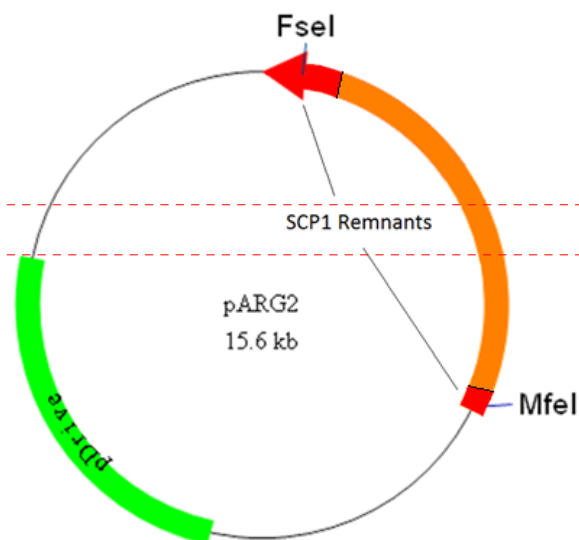
The Metacaspase-1 Knockout

It was decided that the $\Delta Ku80$ knockout-ready strain obtained from the Dutch research group should be used in the generation of a Metacaspase-1 knockout organism for the purpose of investigating Metacaspase function. The general procedure was similar to the one followed in the attempt to generate a *Ku70* knockout; the process was initiated with the creation of the knockout construct transformation vector. This step was accomplished by a previous research student, (Goldberg, 2009) and the work described in this report begins immediately after the generation of the vector. It is important to note that unlike the *Ku70* knockout construct, the *Scp1* knockout construct was not created by the insertion of foreign non-coding DNA; in this case the *Scp1* gene was disrupted by removing a small segment of its coding region and inserting a functional copy of the *Trp1* gene, which conveys the ability to synthesize the essential amino acid tryptophan.

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Figure 6. The *Scp1* knockout construct, pARG2. The function of the *Scp1* gene was disrupted by the insertion of the *Trp1* gene (orange), which conveys the ability to synthesize tryptophan, an essential amino acid. From (Goldberg, 2009)



This induced ability was used as a selectable marker in the later screening phase, by growing transformants in a media which lacked tryptophan it was possible to ensure that only those individuals that took up the plasmid were able to proliferate.

Transformation into *S. commune*

In order to introduce the modified *Scp1* construct to the organism, the circular vector was linearized and transformed into *S. commune* protoplasts. The vector was linearized in some cases by digestion with the enzyme Xba1, which had been previously determined to cut the vector in two locations while leaving the coding region of the construct intact and leaving an appropriate amount of flanking DNA. This DNA segment was then transformed into protoplasts of *S. commune* using the procedure described above. Protoplasts generated from three $\Delta Ku80$ strains were used as transformation recipients: strains 33, 14 and 15. The transformants generated from strains 33 and 15 made use of non-linearized pARG2, as did one of the transformants generated from strain

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14; the remaining transformants used linearized vector. For both cut and uncut vectors, the expectation was that a small number of transformation recipients would integrate the modified *ScpI* construct through a double-crossover event. Since the $\Delta ScpI$ construct was being transformed into the $\Delta Ku80$ strain obtained from the de Jong lab, theoretically, any $\Delta ScpI$ construct which was integrated into the genome of a protoplast would be forced to integrate homologously, resulting in the removal of the functional copy of *ScpI* and its replacement by the construct.

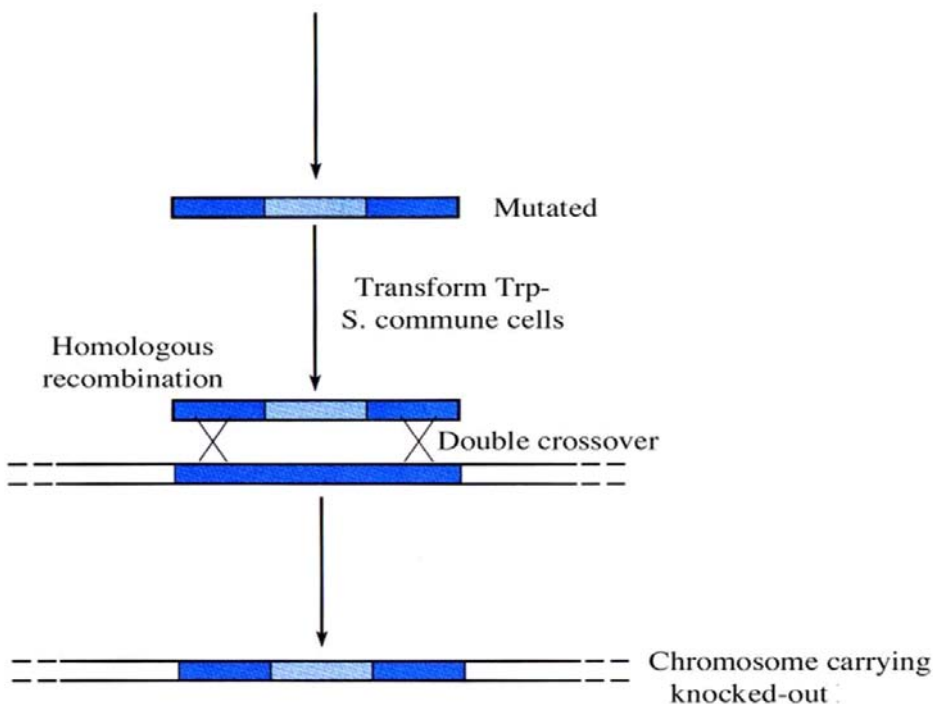


Figure 7. The integration of the $\Delta ScpI$ construct. Integration must occur in a “like-seeks-like” manner, causing the guaranteed replacement of the functional *ScpI* gene with its non-functional experimental counterpart. Since this transformation was taking place in homokaryotic protoplasts, this double-crossover event needed to happen only once per cell to generate a true knockout individual.

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Following transformation, the fungal cells were plated on a CYM plate lacking tryptophan and incubated at 37 °C for several days to allow the individuals to proliferate into macroscopically observable colonies.

Screening the Transformation Recipients

Previous attempts at screening transformation recipients for the presence of a true *Scp1* knockout were particularly laborious as they required the use of traditional DNA isolation techniques as well as the analysis of a greater number of colonies. The use of the *ΔKu80* strain reduced the number of successful transformants, but increased the likelihood that any particular colony was a true knockout. Four Trp⁺ transformants were selected for additional screening; these transformants were Meta-3 5-1, 6-1, 6-2 and 6-3. A 2mm plug was cut from each of these strains and subjected to the Microwave Prep DNA isolation technique described in the Methods section; DNA isolated in this manner was used as the template in subsequent PCR screening experiments. DNA concentrations from each of these preps was tested with the Nanodrop machine, results are shown below in Table 2.

Strain	ng/μl	260/280
Sko2-14 (control)	24.7	1.87
Δmeta 5-1	1759.8	2.15
Δmeta 6-1	1882.8	2.15
Δmeta 6-2	1320.2	2.18
Δmeta 6-3	1076.9	2.18

Table 2. DNA concentrations of the transformation recipients selected for additional screening. DNA concentrations were obtained by means of the Nanodrop machine described above. “Δ meta” refers to transformation recipients before they had had their knockout status confirmed.

DNA concentrations of the samples obtained from the transformation recipients were determined to be too high for use in PCR, so the samples were diluted 1:10 in TE buffer.

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Negative Knockout Confirmation by PCR

At the time of the generation of these transformants, primers to isolate the knockout version of the *Scp1* gene had not been designed, so in order to gain preliminary information about the status of the knockout, a negative confirmation experiment was designed. Each of the four potential knockouts were subjected to a PCR using primers specific to the unmodified version of the *Scp1* gene. These primers were designed to anneal to the gene within the region that was removed in the modified version, so a true knockout individual would be expected to be completely unable to produce a band on an agarose gel.

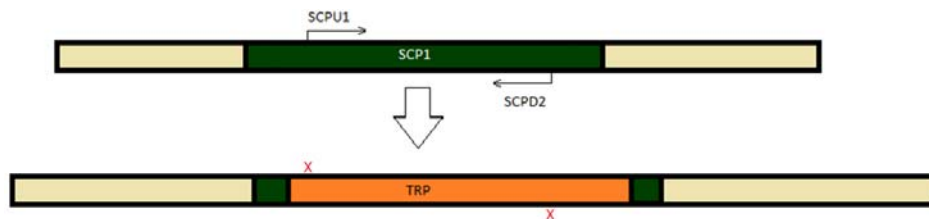


Figure 8. Primer pairs SCPKO U1/D2, U3/D4, and U5/D6 were designed to isolate the unmodified version of *Scp1*. The insertion of the Trp gene, done in the creation of the knockout construct, disrupted the annealing sites for these particular primer pairs, making it impossible for them to amplify the modified *Scp1*. These primer pairs were used in the confirmation of the knockout to show that candidates did not have a functional copy of the *Scp1* gene in their genome.

DNA preps from the four potential knockout were mixed with three primer pairs: SCPKO_U1/D2, SCPKO_U3/D4 and SCPKO_U5/D6. The results of this initial PCR are shown below in Figure 8.

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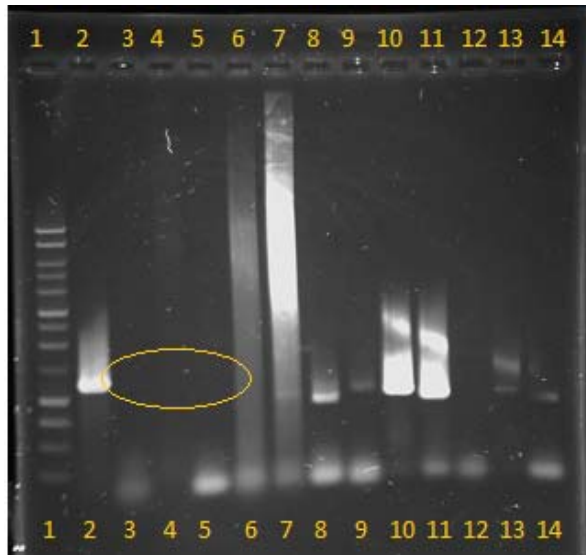


Figure 9. Agarose gel showing the results of the negative confirmation PCR. Absence of bands in lanes 3-5 indicate the inability of the primers to isolate a functional *Scp1* gene. Lanes 3-5 correspond to transformant Δ meta 5-1, implying potential knockout status. The lack of a band in lane 12 is likely not indicative of a knockout as there are faint bands in lanes 13 and 14, and all three off these lanes contain the same genomic DNA.

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In Figure 8, lanes 3-5 correspond to transformant 5-1, lanes 6-8 correspond to transformant 6-1, lanes 9-11 correspond to transformant 6-2, and lanes 12-14 correspond to transformant 6-3. In the lanes corresponding to transformants 6-1, 6-2, and 6-3 there was a band around 1 kb, indicating the successful isolation of a functional *Scp1* gene, and therefor indicating the absence of a knockout. However, lanes 3-5 were observed to be completely blank, indicating that transformant 5-1 may be a true knockout, as it appears to no longer possess a functional copy of *Scp1*. There were concerns that the lack of bands in these lanes was the result of a poor quality genomic template and was not indicative of the presence of a knockout, for this reason a control gel was run to demonstrate the viability of the genomic DNA templates.

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In order to confirm the viability of the PCR primers and DNA templates used in the negative confirmation experiment, a control PCR was run. All three of the above primer pairs were used to detect the presence of *Scp1* in the Sko2-14 strain of *S. commune* which was known to have an intact version of the gene. Furthermore, each of

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the four potential knockouts were subjected to amplification by primers specific to the *STS1* gene, a sugar transporter gene which was not modified in this study. As a control, the Sko2-14 DNA template was also subjected to amplification by STS-specific primers.

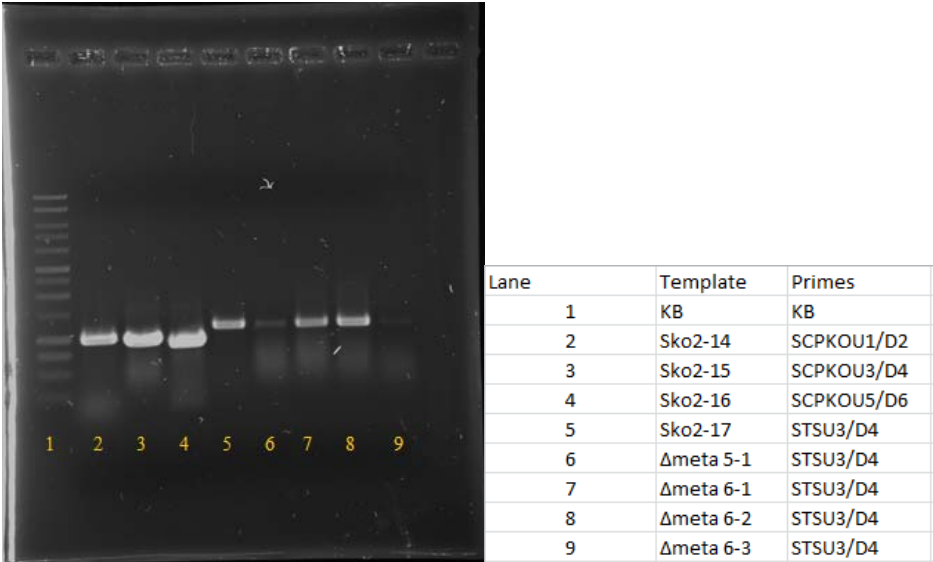


Figure 10. Image of the agarose gel corresponding to the control PCR. Bands in lanes 2-4 indicate that all three of the primer pairs used in the negative screening will successfully isolate an unmodified *Scp1* gene if it is present in the sample. Band in lane 5 indicates that the *STS1* primers will successfully amplify an intact *STS1* gene if it is present in the sample. Lanes 6-9 indicate that all of genomic DNA samples isolated from the potential knockouts are of high enough quality to serve as a template for PCR, as they were used in the successful isolation of *STS1*. The varying intensities of the bands indicates that the degree to which the gene is amplified depends on the initial concentration of the template.

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The presence of bands around 1000 bp in lanes 2-4 of the control gel indicate that all three of the SCPKO primer pairs will successfully isolate the intact *Scp1* gene if it is present in the genomic DNA sample. The presence of bands in lanes 6-9 indicates that all four of the genomic DNA samples isolated from the potential knockouts are of high enough quality to be used as templates for PCR. These results further supported the assumption that the lack of bands in lanes 3-5 of the negative confirmation gel were likely the result of transformant 5-1 being a knockout individual rather than as a result of

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poor DNA template quality. At this point it was decided that transformant 5-1 was a

serious candidate for a *Scp1* knockout, and primers were designed to specifically isolate and amplify the modified version of *Scp1* for the purpose of confirming its presence in the genome of strain Δ meta 5-1.

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Positive Knockout Confirmation by PCR

In order to conclusively demonstrate that transformant 5-1 was a true knockout, primers were designed which would anneal to both the modified and unmodified version of the *Scp1* gene. Specifically, these primers annealed to the *Scp1* remnants flanking the *TrpI* insert. Since the *TrpI* insert (4.5 kb) is about 1kb longer than the piece of *Scp1* that was removed in the formation of the knockout construct the primers could be used to distinguish between the two versions of the gene on basis of size. Initial attempts to use this experimental approach have not proven successful, likely due to lack of PCR optimization.

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Figure 11. General representation of the principle used in the creation of the positive confirmation primers. As shown, the primers anneal outside of the modified region of the gene, allowing them to amplify both the intact *Scp1* and the modified construct. Since the insertion of the *TrpI* insert (4.5 kb) results in the modified version of the gene to be about 1 kb larger than the unmodified version, gel electrophoresis can be used to distinguish between the two versions of the gene.

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Generation of a Dikaryon

In order to observe *S. commune* throughout its lifecycle to detect any phenotypic effects of the *Scp1* knockout a dikaryon individual must first be generated; this individual must be a double-knockout, possessing two copies of the modified *Scp1* gene. This will be achieved through traditional genetic techniques which will allow us to manipulate the heredity of the strain.

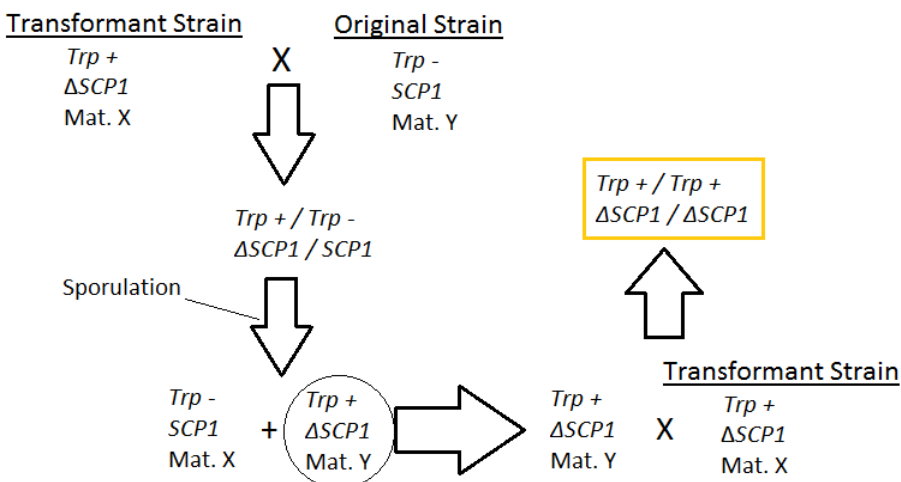


Figure 12. Flowchart diagramming the future genetics work necessary to generate a dikaryon double-knockout individual.

In order to generate a dikaryon individual, the knockout homokaryon was mated with an unmodified individual of the original strain, but of a different mating type. This mating generated a dikaryon with one intact and one null version of the *Scp1* gene. This dikaryon individual was allowed to sporulate, with the hopes that some of the spores would possess the null version of the gene, but would be of a different mating type than the null used in the first step through random gametic variation. In future work, this spore, of mating type “Y”, could then be mated with the original transformant strain, generating a dikaryon individual with two null versions of *Scp1*. This homozygous null

individual would then be allowed to proceed through its life cycle, and observation will be made of any deviances from the typical mushroom development. From these developmental observations it is hoped that some inference about the function of the *Scp1* gene will be able to be made.

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